

Perinatal germ cell development and differentiation in the male marmoset (*Callithrix jacchus*): similarities with the human and differences from the rat

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STUDY QUESTION: Is perinatal germ cell (GC) differentiation in the marmoset similar to that in the human?

SUMMARY ANSWER: In a process comparable with the human, marmoset GC differentiate rapidly after birth, losing OCT4 expression after 5–7 weeks of age during mini-puberty.

WHAT IS KNOWN ALREADY: Most of our understanding about perinatal GC development derives from rodents, in which all gonocytes (undifferentiated GC) co-ordinately lose expression of the pluripotency factor OCT4 and stop proliferating in late gestation. Then after birth these differentiated GC migrate to the basal lamina and resume proliferation prior to the onset of spermatogenesis. In humans, fetal GC differentiation occurs gradually and asynchronously and OCT4⁺ GC persist into perinatal life. Failure to switch off OCT4 in GC perinatally can lead to development of carcinoma *in situ* (CIS), the precursor of testicular germ cell cancer (TGCC), for which there is no animal model. Marmosets show similarities to the human, but systematic evaluation of perinatal GC development in this species is lacking. Similarity, especially for loss of OCT4 expression, would support use of the marmoset as a model for the human and for studying CIS origins.

STUDY DESIGN, SIZE AND DURATION: Testis tissues were obtained from marmosets ($n = 4–10$ per age) at 12–17 weeks' gestation and post-natal weeks 0.5, 2.5, 5–7, 14 and 22 weeks, humans at 15–18 weeks' gestation ($n = 5$) and 4–5 weeks of age ($n = 4$) and rats at embryonic day 21.5 (e21.5) ($n = 3$) and post-natal days 4, 6 and 8 ($n = 4$ each).

PARTICIPANTS/MATERIALS, SETTING AND METHODS: Testis sections from fetal and post-natal marmosets, humans and rats were collected and immunostained for OCT4 and VASA to identify undifferentiated and differentiated GC, respectively, and for Ki67, to identify proliferating GC. Stereological quantification of GC numbers, differentiation (% OCT4⁺ GC) and proliferation were performed in perinatal marmosets and humans. Quantification of GC position within seminiferous cords was performed in marmosets, humans and rats.

MAIN RESULTS AND ROLE OF CHANCE: The total GC number increased 17-fold from birth to 22 post-natal weeks in marmosets; OCT4⁺ and VASA⁺ GC proliferated equally in late gestation and early post-natal life. The percentage of OCT4⁺ GC fell from 54% in late fetal life to <0.5% at 2.5 weeks of age and none were detected after 5–7 weeks in marmosets. In humans, the percentage of OCT4⁺ GC also declined markedly during the equivalent period. In marmosets, GC had begun migrating to the base of seminiferous cords at ~22 weeks of age, after the loss of GC OCT4 expression.

LIMITATIONS, REASONS FOR CAUTION: There is considerable individual variation between marmosets. Although GC development in marmosets and humans was similar, there are differences with respect to proliferation during fetal life. The number of human samples was limited.

WIDER IMPLICATIONS OF THE FINDINGS: The similarities in testicular GC differentiation between marmosets and humans during the perinatal period, and their differences from rodents, suggest that the marmoset may be a useful model for studying the origins of CIS, with relevance for the study of TGCC.

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Key words: testis / fetal / post-natal / germ cell / differentiation

Introduction

The male common marmoset (*Callithrix jacchus*) has been proposed as a more appropriate model than rodents for study of human testicular function, in particular for studies on fetal and neonatal germ cell (GC) development and differentiation. The marmoset and human are broadly similar in terms of overall testis development and function. Both species exhibit a neonatal surge in gonadotrophin and reproductive hormonal activity, now generally termed as 'mini-puberty'. This lasts from birth until 12–15 weeks of age in the marmoset, with a peak in plasma testosterone levels at around 2–3 weeks of age (Dixon, 1986; Lunn *et al.*, 1994; McKinnell *et al.*, 2001), and from 1 month until 4–6 months of age in the human, peaking at around 2 months of age (Forest *et al.*, 1974; Job *et al.*, 1988; Gustafson *et al.*, 1993). This is followed by an extended period of 'childhood' quiescence (Chemes, 2001; Kelnar *et al.*, 2002), which does not occur in the rodent (Plant, 2006). In addition, subsequent organization and efficiency (number of GC supported per Sertoli cell) of spermatogenesis is similar in adult marmosets and humans but notably different in rodents (Sharpe, 1994; Millar *et al.*, 2000; Sharpe *et al.*, 2000; Weinbauer *et al.*, 2001).

There are other significant differences between rodents and primates, particularly in relation to GC development during perinatal life. In the rat, gonocytes (undifferentiated GC) enter a period of proliferative quiescence at around e17–e18 and do not recommence proliferation until a few days after birth (Moréno *et al.*, 2001; Ferrara *et al.*, 2006; Jobling *et al.*, 2011). Also, GC differentiation in rodents is synchronous; for example, all GC in the rat coordinately lose expression of the pluripotency marker OCT4, so that by e19 OCT4-positive GC are absent (Ferrara *et al.*, 2006; Jobling *et al.*, 2011). In contrast, in humans and marmosets there is no stage of fetal GC proliferative quiescence (Honecker *et al.*, 2004; Mitchell *et al.*, 2008), and the fetal GC population is heterogeneous and differentiates asynchronously throughout fetal life and the neonatal period (Gaskell *et al.*, 2004; Mitchell *et al.*, 2008; McKinnell *et al.*, 2009). Thus, individual seminiferous cords contain undifferentiated (OCT4 positive/VASA negative) and differentiated (OCT4 negative/VASA positive) GC side by side from mid-gestation until the first few weeks of life. However, there are conflicting data from the marmoset regarding the precise degree to which expression of OCT4 and other markers of undifferentiated GC persists after birth. Studies from our own laboratory suggest that pluripotency factors OCT4 and NANOG and the transcription factor AP2gamma share a very similar pattern of GC expression, all declining gradually during the first 6 weeks after birth (Mitchell *et al.*, 2008). In contrast, Albert *et al.* (2010) reported that the GC population expressing OCT4 and AP2gamma is stable, with numbers remaining constant until at least 8 weeks of age. This question is of particular

importance because OCT4, AP2gamma and NANOG are also markers of carcinoma *in situ* (CIS), the precursor of testicular germ cell cancer (TGCC) in humans (Rajpert-de Meyts, 2006). It is thought that CIS arises because of a failure of some GC to switch off expression of such undifferentiated GC factors (Rajpert-de Meyts, 2006), and the asynchronous nature of GC differentiation in humans may predispose towards this. The incidence of TGCC is increasing in Western countries (Bray *et al.*, 2006), but investigation of potential factors underlying its occurrence is hampered by poor understanding of perinatal GC development (Culty, 2009) and the lack of an appropriate animal model.

Most of our understanding about regulation of GC development and differentiation derives from rodents while relatively little is known in primates. As a consequence, the default assumption is that GC development occurs as in rodents. For example, at around post-natal day (pnd) 6 in rats, GC migrate from the centre of seminiferous cords to make contact with the basal lamina, a step which is essential for their differentiation into spermatogonia and for proliferation to recommence (Roosen-Runge and Leik, 1968; McGuinness and Orth, 1992). It is assumed that the same process is required in primates, but when this occurs is unclear, nor is it known for certain whether GC migration occurs subsequent to the loss of expression of pluripotency markers, as in the rodent, or whether these two aspects of differentiation overlap. This question is of interest in the context of CIS/TGCC, because in the normal human testis during the perinatal period, those GC which remain OCT4 positive are almost exclusively located in the centre of the seminiferous cords (Honecker *et al.*, 2004; Cools *et al.*, 2005). On the other hand, in older children with disorders of sexual development or undervirilization, who are at increased risk of TGCC, GC expressing OCT4 are reported to be both more numerous and located at the basal lamina (Cools *et al.*, 2005; Hersmus *et al.*, 2012). The marmoset may be a human-relevant model in which to address these issues. A number of studies have analysed GC number, differentiation (Sharpe *et al.*, 2000, 2002, 2003; McKinnell *et al.*, 2009; Albert *et al.*, 2010) and proliferation (Kelnar *et al.*, 2002; Mitchell *et al.*, 2008; McKinnell *et al.*, 2009) at specific timepoints during perinatal life in the marmoset, but the conflicting marmoset data mentioned above highlight the need for a comprehensive quantitative study of these parameters spanning the period from fetal life through to the end of mini-puberty. The aims of the present study were therefore: firstly, to analyse and delineate more precisely the normal course of male GC development in the marmoset during this period, with regard to proliferation, differentiation and migration; secondly, using OCT4 expression as a marker, to address in particular the inconsistency in existing data regarding the persistence of undifferentiated GC after birth; and thirdly, by comparison with the human and the rat, to evaluate whether or not the marmoset is a potentially suitable model

for the human, especially in relation to the perinatal origins of CIS, the precursor of TGCC.

Materials and Methods

Animals

Common marmoset monkeys (*Callithrix jacchus*) were captive-bred and maintained in a closed colony that has been self-sustaining since 1973. Wistar rats were maintained in our own animal facility according to UK Home Office guidelines. Studies were performed according to the Animal (Scientific Procedures) Act 1986 under UK Home Office Project Licence approval, and also approved by the local ethical committee for studies in primates.

Tissue collection and processing

Marmoset fetal testes

Testes were obtained from the fetuses of pregnant marmosets in the colony breeding stock that were being euthanized for colony management purposes. Gestation in the marmoset lasts for 144 days (Windle et al., 1999). The gestation of pregnant females was assessed by systematic palpation and/or ultrasound. Experience has shown this to be accurate within 1 week and was subsequently confirmed by time of birth. Mothers and fetuses were euthanized by injection of an overdose of sodium pentobarbitone (Euthatal; Rhone Merieux Ltd, Harlow, Essex, UK). The fetuses (12–17 weeks, $n = 5$) were delivered by hysterotomy. Fetuses were fixed in Bouins for 6 h (larger fetuses were partially dissected prior to fixing) and then transferred to 70% ethanol.

Marmoset post-natal testes

Testes at ~0.5 weeks (1–5 days, $n = 10$), ~2.5 weeks (17–20 days, $n = 5$), 5–7 weeks ($n = 9$), 14 weeks ($n = 4$) and 22 weeks ($n = 6$) of age were from control animals that had been used for previous studies, in order to reduce the animal numbers required (Lunn et al., 1997; Kelnar et al., 2002; Sharpe et al., 2002, 2003; McKinnell et al., 2009); in addition, some testes were from animals euthanized for colony management purposes. All testes were fixed as described above, prior to processing.

Human fetal testes

Testes were obtained following termination of pregnancy at 15–18 weeks' gestation ($n = 5$). Women gave consent in accordance with national guidelines (Polkinghorne, 1989), and ethical approval was obtained from the Local Research Ethics Committee. No terminations were due to fetal abnormalities. Gestational age was determined initially by ultrasound examination, followed by direct measurement of foot length. Testes were fixed for 2 h in Bouins and then transferred into 70% ethanol prior to processing.

Human post-natal testes

Testes were obtained at autopsy with consent of their legal guardian (courtesy of K. McKenzie, Department of Pathology, Royal Infirmary of Edinburgh) from boys ($n = 4$) who died at 4–5 weeks of age from various causes (excluding reproductive and endocrine abnormalities). Testes were fixed in 10% neutral-buffered formalin for at least 24 h and processed as below.

Rat fetal testes

Pregnant dams were killed by inhalation of carbon dioxide when their fetuses had reached e21.5 ($n = 3$). Fetuses were removed, decapitated and placed in ice-cold phosphate-buffered saline (PBS; Sigma, Poole,

UK). Testes were removed via microdissection and fixed for 1 h in Bouins followed by transfer into 70% ethanol.

Rat post-natal testes

Male rats aged 4, 6 and 8 days ($n = 4$ at each age) were anaesthetized by CO₂ inhalation and then killed by cervical dislocation. Testes were removed, fixed for 6 h in Bouins and transferred into 70% ethanol.

Tissue processing

All fixed testes were embedded in paraffin wax using standard methods in an automatic processor (Leica Microsystems, Milton Keynes, UK) and sections of 5 μ m thickness were prepared.

Immunohistochemistry

Specific proteins were detected by immunohistochemistry using methods detailed previously (Gaskell et al., 2004; Ferrara et al., 2006; Mitchell et al., 2008). An antibody against VASA (ab13840; Abcam, Cambridge, UK) was used at a dilution of 1:200; antibodies against OCT4 (sc-8628; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Ki67 (M7240; Dako, Ely, UK) were used at 1:40. Briefly, antigen retrieval used 0.01 M Citrate buffer, pH 6.0. Endogenous peroxidase was blocked by incubating slides for 30 min in 3% (vol/vol) H₂O₂ in methanol and endogenous biotin was blocked using a streptavidin/biotin blocking kit (Vector Laboratories Inc., Peterborough, UK) according to the manufacturer's instructions. Non-specific binding was blocked by incubating slides in appropriate normal serum diluted 1:5 in TBS containing 5% (w/v) bovine serum albumen (BSA; Sigma). After incubation overnight at 4°C with primary antibody, slides were washed in TBS then incubated with appropriate biotinylated secondary antibody, followed by incubation with streptavidin-conjugated horseradish peroxidase (Dako) and visualization of immunostaining using diaminobenzidine (Liquid DAB+; Dako).

Double immunostaining using fast blue visualization

VASA/OCT4

After incubation with the primary (VASA) and secondary antibodies as described above, slides were washed in TBS, incubated with streptavidin-conjugated alkaline phosphatase (Dako) diluted 1:200 in TBS, and immunostaining was visualized using 1 mg/ml Fast Blue (Sigma-Aldrich Ltd, Poole, UK) until staining was optimal. After washing in TBS, slides were immunostained for OCT4 using DAB visualization as described above.

Ki67/VASA

Immunostaining for the proliferation marker Ki67, using DAB detection, was performed as described above. Slides were then washed in TBS and immunostained for VASA using Fast Blue as described above.

To ensure reproducibility of results and accurate comparison of immunostaining, sections from all groups were run in parallel on at least two occasions. Negative controls were included in each experiment, for which the primary antibody was replaced with either peptide-preabsorbed antibody (OCT4) or the appropriate normal serum (VASA, Ki67) and, in all cases, immunostaining was absent. Representative sections were photographed using a Provis AX70 microscope (Olympus Optical, London, UK) fitted with a Canon DS6031 digital camera (Canon Europe, Amsterdam, The Netherlands). Images were compiled using Photoshop Elements 9 (Adobe Systems Inc., Mountain View, CA, USA).

Triple immunofluorescence

Details of antibodies and detection reagents are shown in Table I. Antigen retrieval, blocking and incubation with the first primary antibody were

Table 1 Antibodies and conditions for triple immunofluorescence.

Antigen	Dilution	Secondary antibody	Visualization
OCT4	1:100	^a CAG-p	^d Tyr-Cy3
VASA	1:100	^b CAR-p	^e Tyr Fluor
Ki67	1:100	^c CAM-p	^f Tyr-Cy5

^aCAG-p—Chicken anti-goat peroxidase (Santa Cruz Biotechnology, CA, USA).

^bCAR-p—Chicken anti-rabbit peroxidase (Santa Cruz).

^cCAM-p—Chicken anti-mouse peroxidase (Santa Cruz).

^dTyr-Cy3—Tyramide Cy3 (Perkin Elmer, MA, USA).

^eTyr Fluor—Tyramide Fluorescein (Perkin Elmer).

^fTyr-Cy5—Tyramide Cy5 (Perkin Elmer).

performed as described above for immunohistochemistry. Sections were incubated with a peroxidase-conjugated secondary antibody diluted in normal serum/TBS/BSA for 30 min and kept in the dark thereafter. Slides were then incubated with labelled Tyramide diluted in buffer at 1:50 for 10 min before being placed in boiling citrate buffer and micro-waved on full power for 2.5 min and left to cool for a further 30 min. The incubations with primary antibody, peroxidase-conjugated secondary antibody and Tyramide (with different fluorescent labels) were then repeated for the second and third primary antibodies. Following incubation of the second Tyramide label, sections were incubated for 15 min in 3% (vol/vol) H₂O₂ in TBS to block any remaining peroxidase. Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) diluted 1:1000 in PBS for 10 min. Slides were mounted using Permafluor (Immunotech, Marseille, France). Slides were scanned and tiled images of complete testis sections were captured using an LSM 710 confocal microscope and ZEN 2009 software (Carl Zeiss, Hertfordshire, UK).

Determination of GC numbers

Sections were analysed using a Zeiss Axio-Imager microscope (Carl Zeiss Ltd, Welwyn Garden City, UK) fitted with a Hitachi HV-C20 camera (Hitachi Denshi Europe, Leeds, UK) and a Prior automatic stage (Prior Scientific Instruments Ltd, Cambridge, UK). Image-Pro 6.2 with Stereologer plug-in software (MagWorldwide, Wokingham, UK) was used to select random fields for counting and to place a grid over the tissue. GC counting used sections immunostained for VASA or OCT4. Relative cell volume per testis was first determined by point counting (Sharpe *et al.*, 2003). The number of fields counted per animal (~15–75 fields) was dependent on obtaining a percentage SE value of <5%. Data were converted to absolute volume per testis by multiplying by testis weight (equivalent to volume), then converted to cell number per testis after determination of mean cell nuclear diameter and volume (average of ~100 nuclei) using the Stereologer software nucleator function.

Determination of the GC proliferation index in the marmoset

GC proliferation in fetal and 0.5-week-old marmoset testes was quantified using sections subjected to triple immunofluorescence for OCT4/VASA/Ki67, which enabled identification of proliferating and non-proliferating cells within both OCT4⁺ and VASA⁺ GC subpopulations. Confocal images of complete testis cross-sections (2–3 per animal) were examined using AxioVision 4.8 software (Carl Zeiss), and all OCT4⁺/Ki67⁺, OCT4⁺/Ki67⁻, VASA⁺/Ki67⁺ and VASA⁺/Ki67⁻ cells were counted. For both OCT4⁺ and VASA⁺ GC subpopulations, proliferation index

(PI) was calculated as the number of cells which were also Ki67⁺ in each subpopulation divided by the total number of each subpopulation, counted $\times 100$. For marmosets at 14 and 22 weeks of age, the simpler method of VASA/Ki67 double immunostaining was used, as only VASA⁺ GC were present. Stereology was used to select 15–20 fields at random from two sections per animal, and a total of 250–300 GC were counted. The PI was calculated as the number of VASA⁺/Ki67⁺ cells divided by the number of Ki67⁺/VASA⁺ plus VASA⁺/Ki67⁻ cells $\times 100$.

Percentage of GCs expressing OCT4 in the marmoset and human

Using the data for GC numbers determined as described earlier, the percentage of OCT4⁺ GC in post-natal marmosets was calculated as the number of OCT4⁺ cells divided by the total number of GC (OCT4⁺ plus VASA⁺), $\times 100$. In fetal marmosets and in the human, we were unable to calculate absolute numbers of GC as data for testis volume/weight was unavailable. We therefore used stereology to analyse sections double-immunostained for OCT4 and VASA (fetal marmosets) or sections immunostained for OCT4 only (humans). In the latter, we identified immunonegative GC on the basis of their morphology. We used stereology to select random fields, and counted ~200 GC from 2–3 sections per individual, then determined the proportion of OCT4⁺/VASA⁻ or OCT4⁺ cells as a percentage of total GC counted.

Determination of GC position in the marmoset, human and rat

As a measure of GC development, we analysed the GC complement in terms of their position within the seminiferous cords and to compare the marmoset, human and the rat for this parameter. On pairs of serial sections immunostained for VASA and OCT4, we identified GC that were centrally located (no part of the cell having any contact with the basal lamina), in partial contact with the basal lamina (only a small part of cell cytoplasm in contact), and basally located (with the cell nucleus and/or a large part of the cytoplasm in close contact with the basal lamina). These criteria are illustrated in Fig. 1. We used stereology to select random fields and a minimum of 150 VASA⁺ cells, from two sections per testis, were counted at each age. For testes from fetal and 0.5-week-old marmosets and from the fetal human, we also counted a minimum of 150 OCT4⁺ cells, from two sections per testis. However, in marmosets at 2.5 weeks and 5–7 weeks of age, because OCT4⁺ cells were much fewer in number, we used sections from all animals and several immunostaining runs in order to count a pooled total ~55 cells at both these ages. For each GC subpopulation, the proportion of cells in each position was then calculated as a percentage of the total number of VASA⁺ or OCT4⁺ cells counted.

Statistical analysis

Data were analysed using Student's *t*-test (two-tailed), except for between-age comparisons of data for GC position, for which one-way analysis of variance was used.

Results

GC numbers in the marmoset

Fetal marmoset and human testes contain a heterogeneous population of undifferentiated and differentiated GC (Gaskell *et al.*, 2004; Mitchell *et al.*, 2008), with some undifferentiated GC persisting into neonatal life (Mitchell *et al.*, 2008; McKinnell *et al.*, 2009). We have previously

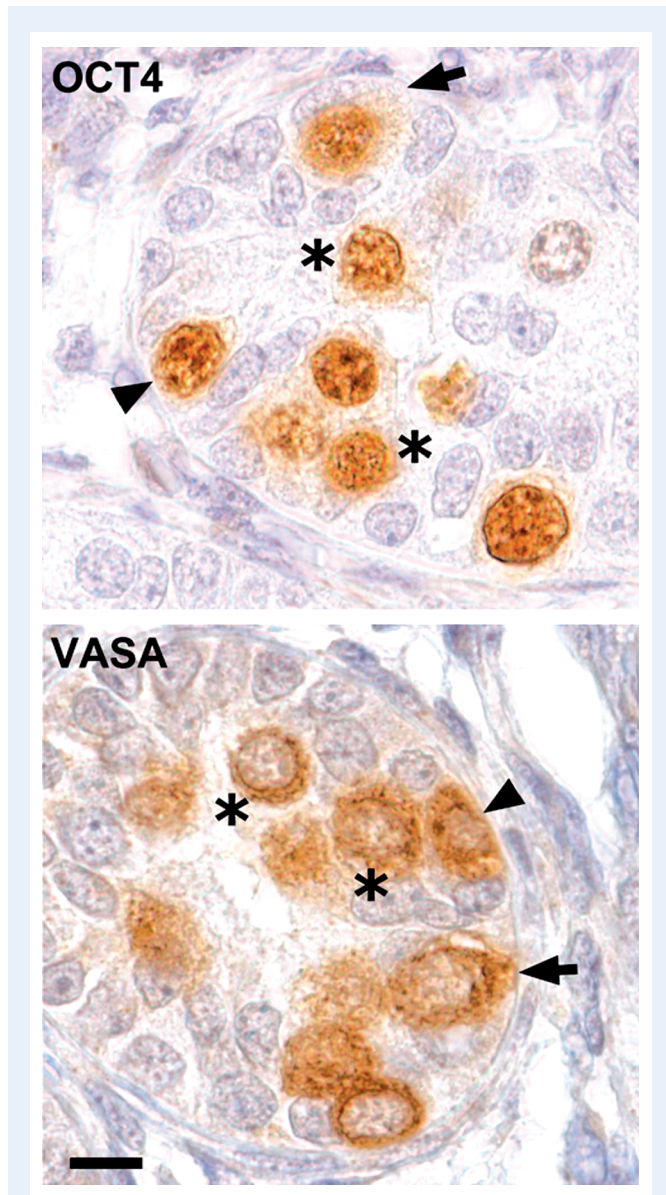


Figure 1 Photomicrograph of post-natal marmoset testes immunostained for OCT4 or VASA to illustrate the criteria used to identify germ cells which are centrally located within seminiferous cords (asterisks; no part of the cell having any contact with the basal lamina), in partial contact with the basal lamina (arrows; only a small part of cell cytoplasm in contact) and basally located (arrowheads; the cell nucleus and/or a large part of the cytoplasm in close contact with the basal lamina).

published data for total GC numbers, i.e. undifferentiated (OCT4^+) plus differentiated (VASA^+) GC, in 1–5-day-old (0.5 week) marmosets and the number of VASA^+ GC at 17–20 days (2.5 weeks) of age (McKinnell et al., 2009). In order to more systematically investigate the dynamics and differentiation of the GC population in marmosets throughout the neonatal period, we have now examined OCT4 and VASA immunorexpression at 2.5, 5–7, 14 and at 22 weeks of age (Fig. 2) and quantified both VASA^+ and OCT4^+ cells at each age. For the purposes of comparison, we have included the data on GC

numbers from McKinnell et al. (2009) in Table II. Total GC number per testis (OCT4^+ plus VASA^+) at 2.5 weeks was 1.64×10^6 (Table II), an increase of 2.7-fold compared with 0.5 weeks of age. This increase was due entirely to an increase in VASA^+ GC, as numbers of OCT4^+ GC decreased rapidly after birth. Only 0.006×10^6 OCT4^+ GC per testis were present at 2.5 weeks of age (Table II), compared with 0.082×10^6 which we had found previously at 0.5 weeks of age (Table II), and in one out of five animals examined at 2.5 weeks of age no OCT4^+ GC were detected (not shown). Total GC numbers changed only slightly between 2.5 and 5–7 weeks of age, while OCT4^+ GC numbers declined further to 0.004×10^6 per testis at 5–7 weeks (Table II) and in four out of nine animals at this age OCT4^+ GC were undetectable (not shown). Thereafter, no OCT4^+ GC were detected in any animals. Beyond 5–7 weeks, GC numbers increased 1.9-fold to reach 3.77×10^6 by 14 weeks, then rose another 2.5-fold between 14 and 22 weeks of age to 9.76×10^6 (Table II). Between 0.5 and 22 weeks, the GC number increased 17-fold (Table II).

GC proliferation in the marmoset

Since GC proliferation in the human fetal testis is reported to occur predominantly in the OCT4^+ subpopulation (Mitchell et al., 2010), we set out to determine if this is also the case in the marmoset. We used OCT4/VASA/Ki67 triple immunostaining and confocal microscopy to quantify proliferation in OCT4^+ and VASA^+ subpopulations in the fetal and neonatal marmoset (Fig. 3A). While previous studies in fetal and immature marmosets have shown that GC proliferation was present at all timepoints studied up to 6 weeks of age (Mitchell et al., 2008; McKinnell et al., 2009), data for later post-natal ages are lacking. Therefore, the present study also utilized VASA/Ki67 double immunostaining to extend the investigation of GC proliferation to 14 and 22 weeks of post-natal life (Fig. 3A). We found that the overall GC PI in fetal testes was 29.1% (Fig. 3B), and there was no difference in PI between OCT4^+ (28.9%) and VASA^+ (29.9%) subpopulations (Fig. 3B). In the first week of post-natal life, the overall PI declined significantly to 13.3% (Fig. 3B) and again there was no difference in PI between the OCT4^+ (12.2%) and VASA^+ (13.0%) subpopulations. However, the changes in the PI of each subpopulation compared with that in fetal life did not reach statistical significance. By 14 weeks of age only VASA^+ GC were present, and although proliferation was still detectable, the PI was only 2.7%—significantly lower than the overall PI at 0.5 weeks of age (Fig. 3B). Compared with 14 weeks of age, PI increased significantly to 7% at 22 weeks of age (Fig. 3B).

Proportion of OCT4^+ GCs in the marmoset and human testis

We have previously used the percentage of total GC that are OCT4^+ as a quantitative measure of GC differentiation in the marmoset (McKinnell et al., 2009), and in the present study we have further quantified the OCT4^+ GC population in this manner at different ages in order to delineate the progression of differentiation. For the purposes of comparison, the data from McKinnell et al. (2009) are included in Fig. 4. This also allowed us to compare the neonatal GC population in marmosets with that in fetal life (for which a calculation of absolute numbers was not possible) and also to compare fetal and

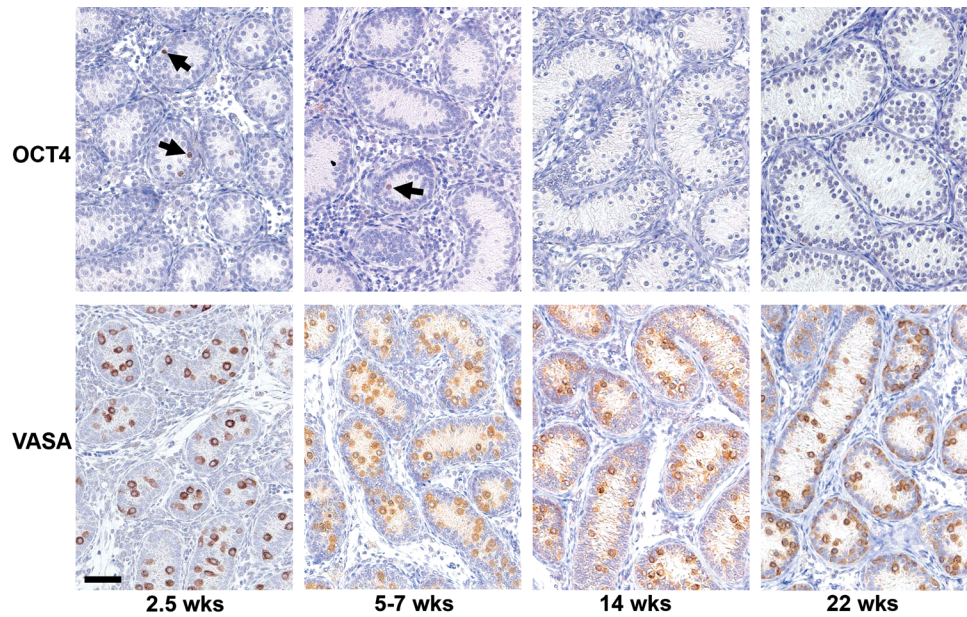


Figure 2 Immunoeexpression of GC markers OCT4 and VASA in testes from post-natal marmosets. OCT4⁺ cells were already rare at 2.5 weeks of age and were not detected in any animals after 5–7 weeks of age. Scale bar = 20 μ m.

Table II Age-related change in numbers (10^6) of VASA⁺ and OCT4⁺ GC in post-natal marmoset testes. No OCT4⁺ cells were detected (ND) after 5–7 weeks of age.

	VASA ⁺	OCT4 ⁺	Total
0.5 weeks ^a	0.494 \pm 0.101	0.082 \pm 0.045	0.576 \pm 0.139
2.5 weeks	1.631 \pm 0.195	0.006 \pm 0.002	1.637 \pm 0.195
5–7 weeks	2.018 \pm 0.331	0.004 \pm 0.002	2.022 \pm 0.330
14 weeks	3.772 \pm 0.735	ND	3.772 \pm 0.735
22 wks	9.765 \pm 1.048	ND	9.765 \pm 1.048

Values are means \pm SEM. 0.5 weeks n = 10; 2.5 weeks n = 5; 5–7 weeks n = 9; 14 weeks n = 4; 22 weeks n = 6.

^aData taken from McKinnell *et al.* (2009).

neonatal marmosets with the human. We found that in marmoset testes at 12–17 weeks' gestation, OCT4⁺ cells comprised almost 54% of the total GC present (Fig. 4). The proportion of GC which were OCT4⁺ at 0.5 weeks was only 12.4% (Fig. 4), indicating a relatively rapid rate of differentiation during late pregnancy and around the time of birth. The proportion of OCT4⁺ GC continued to decline rapidly and significantly, with only 0.4% of GC being OCT4⁺ at 2.5 weeks and 0.3% at 5–7 weeks of age (Fig. 4). After this age, no OCT4⁺ GC were detected in any animals (Fig. 4). In human fetal and neonatal testes, the proportion of OCT4⁺ GC and the progress of differentiation were remarkably similar to the marmoset. We found that OCT4⁺ GC in fetal human testes at 15–18 weeks' gestation comprised almost 62% of the total (Fig. 4), but this declined significantly to \sim 9% by 4–5 weeks after birth (Fig. 4).

GC position in the marmoset, human and rat testis

In rodents, migration of GC to the basal lamina at around the time of birth is considered to be another feature of GC differentiation/maturation (Roosen-Runge and Leik, 1968; McGuinness and Orth, 1992). We used stereology to analyse the GC complement in terms of their position within the seminiferous cords, and to compare the marmoset, human and rat for this parameter. Using criteria illustrated in Fig. 1, we assessed the percentage of the VASA⁺ and OCT4⁺ GC subpopulations that were centrally located, in partial contact with the basal lamina, and basally located. For each species and within each subpopulation, we found that the proportion of GC in partial contact with the basal lamina was relatively constant (12–18% in marmosets and humans, 13–20% in rats) at all timepoints, and for ease of presentation these results are therefore not shown. In considering the following marmoset GC positional data, it should be remembered that the proportion of OCT4⁺ GC declined rapidly after birth with a corresponding increase in the proportion of VASA⁺ GC.

In marmosets at 12–17 weeks of gestation, just under 60% of VASA⁺ GC were centrally located, while \sim 26% were basally located (Fig. 5). Thereafter, the proportion that was centrally located gradually increased and the proportion basally located decreased, so that by 5–7 weeks of age 80% of VASA⁺ GC were centrally located and only 8% were basal; these figures were significantly different from those at 0.5 weeks of age (Fig. 5). The proportions were little changed at 14 weeks of age (Fig. 5). However, at 22 weeks of age the pattern changed dramatically and only 40% of VASA⁺ GC were centrally located, while the same proportion was located basally (Fig. 5). Again, both percentages were significantly different compared with 0.5 weeks of age (Fig. 5). At 12–17 weeks of gestation and at 0.5

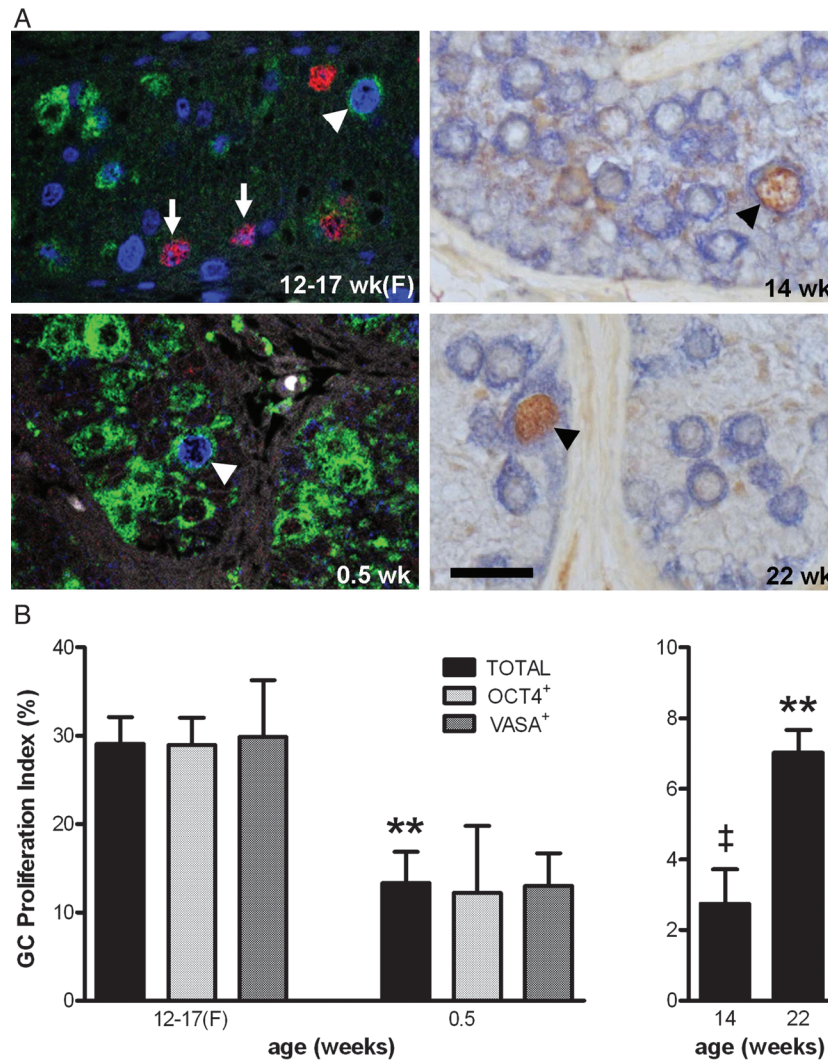


Figure 3 (A) Triple immunofluorescence for OCT4 (red), VASA (green) and Ki67 (blue) was used to identify proliferation in OCT⁺ and VASA⁺ germ cell (GC) subpopulations in fetal (F) marmoset testes at 12–17 weeks' gestation and in post-natal testes at 0.5 weeks of age. Double immunostaining for VASA (blue) and Ki67 (brown) was used to identify proliferating GC at 14 and 22 weeks of age, when OCT4⁺ cells were no longer present. Co-localization of OCT4 and Ki67 in GC nuclei (arrows) and Ki67 staining in nuclei of VASA⁺ GC (arrowheads) is evident. Scale bar = 20 μ m. (B) Overall GC proliferation index (PI) and PI in OCT4⁺ and VASA⁺ GC subpopulations in testes from marmosets at 12–17 weeks' gestation ($n = 5$) and at 0.5 weeks of age ($n = 5$), and in VASA⁺ GC at 14 weeks ($n = 4$) and 22 weeks of age ($n = 6$). Values are means \pm SEM. ** $P < 0.01$ compared with 12–17 weeks (F) or to 14 weeks. ‡ $P < 0.05$ compared with overall PI at 0.5 weeks of age.

weeks of age, OCT4⁺ GC were more evenly distributed between central and basal locations than were the VASA⁺ GC, with ~40% in each location (Fig. 5). However, the proportion of centrally located OCT4⁺ GC then increased rapidly and by 5–7 weeks of age 74% were centrally located and only 12% were basal (Fig. 5), after which OCT4⁺ GC were undetectable.

In the fetal human testis at 15–18 weeks' gestation, in contrast to the fetal marmoset, only a minority (16%) of VASA⁺ GC were centrally located, while just over 70% were basally located (Fig. 5). Conversely, ~55% of OCT4⁺ GC were centrally located and only 33% were basal (Fig. 5).

In the rat testis, OCT4⁺ GC are absent by e19.5 (Ferrara et al., 2006; Jobling et al., 2011), and all GC are VASA⁺ at all ages in the

rat, including when OCT4 is expressed. At e21.5 in the rat almost 80% of GC were centrally located, and <3% were basal, and these proportions remained almost unchanged at pnd 4 (Fig. 5). Compared with 4 days of age, the proportions then changed dramatically and significantly, with over 70% of GC becoming basally located by 8 days of age (Fig. 5), reflecting their migration to the basal membrane. At that age, only 12% of GC remained centrally located (Fig. 5).

Discussion

For reasons set out in the Introduction, the primary goal of the present studies was to characterize testicular GC development (loss of pluripotency, proliferation, location within seminiferous cords)

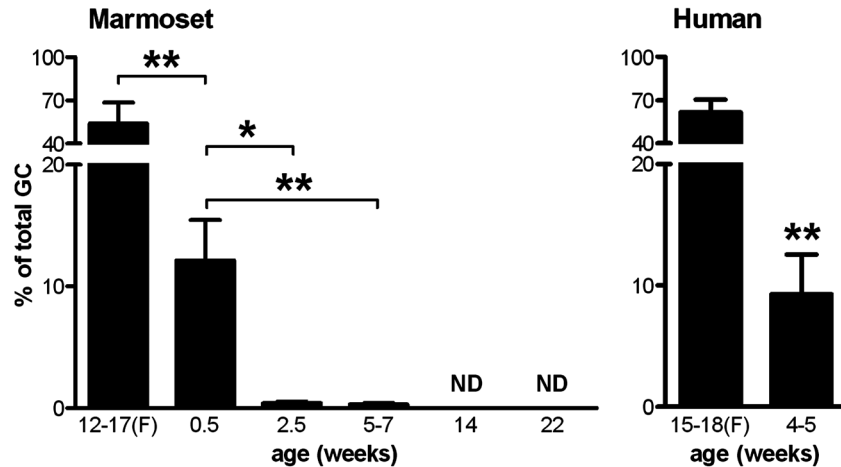


Figure 4 Percentage of total GC number which were OCT4⁺—a measure of GC differentiation—in testes from fetal (F) and post-natal marmosets and humans. Data for 0.5-week-old marmosets is taken from McKinnell *et al.* (2009) and is included for comparison. Values are means ± SEM. Marmosets: 12–17 weeks (F) *n* = 5; 0.5 weeks *n* = 10; 2.5 weeks *n* = 5; 5–7 weeks *n* = 9; 14 weeks *n* = 4; 22 weeks *n* = 6. Human: 15–18 weeks (F) *n* = 5; 4–5 weeks *n* = 4. **P* < 0.05; ***P* < 0.01, in comparison with values at 0.5 weeks (marmosets) or in fetal life (humans).

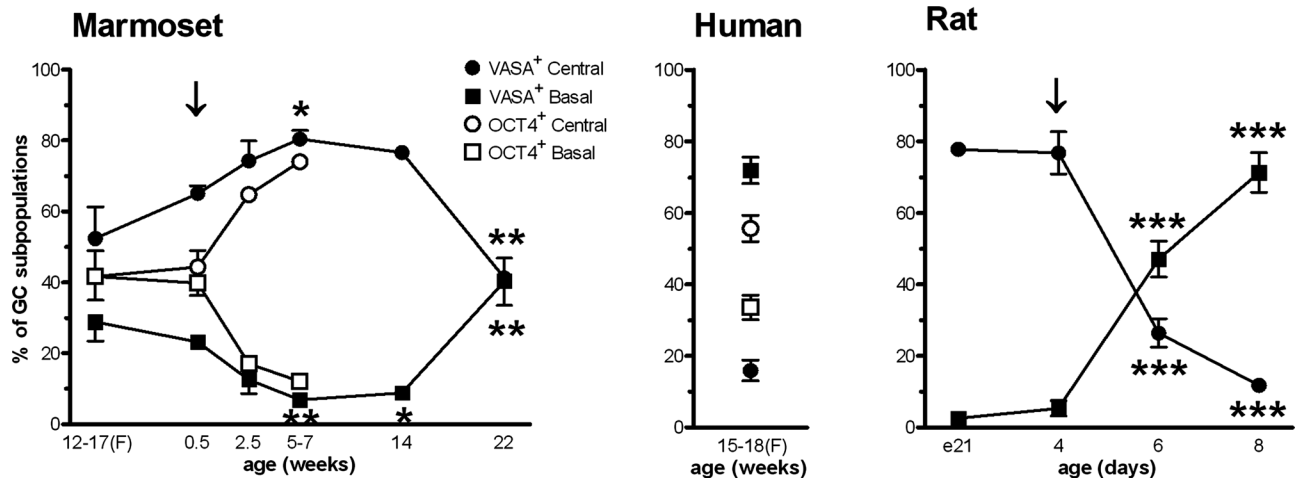


Figure 5 Percentage of OCT4⁺ and VASA⁺ GCs found to be centrally or basally located within seminiferous cords in testes from fetal (F) and post-natal marmoset, fetal human and fetal and post-natal rat. Values are means ± SEM. Marmosets: 12–17 weeks (F) *n* = 5; 0.5 weeks *n* = 9; 2.5 weeks *n* = 5; 5–7 weeks *n* = 5; 14 weeks *n* = 4; 22 weeks *n* = 4. Human: 15–18 weeks (F) *n* = 5; 4–5 weeks *n* = 4. Rat: e21 *n* = 3; 4 days *n* = 4; 6 days *n* = 4; 8 days *n* = 4. In the marmoset and rat, data at other timepoints were compared with 0.5 weeks or 4 days of age respectively (arrows). **P* < 0.05; ***P* < 0.001; ****P* < 0.0001.

from late fetal life beyond the end of mini-puberty in the marmoset, and to compare this with the human and rat. Our results show unequivocally that GC differentiation/loss of pluripotency in marmosets is remarkably similar to that in the human, supporting the use of marmosets for the study of early GC development, for example the perinatal origins of CIS/TGCC in men. Our findings also strongly reaffirm previous conclusions (Ehmcke *et al.*, 2006; Mitchell *et al.*, 2008) that GC development in rodents is fundamentally different from that in the human and marmoset, and more cognizance needs to be taken

of this when extrapolating the results from rodent studies into humans.

In order to investigate the dynamics of GC development, we have extended our earlier study (McKinnell *et al.*, 2009), and quantified both OCT4⁺ and VASA⁺ populations from 2.5 weeks until 22 weeks of age. The former timepoint is the reported peak of mini-puberty in marmosets from our colony, whereas the latter timepoint is after the end of mini-puberty (Lunn *et al.*, 1994; McKinnell *et al.*, 2001). The number of OCT4⁺ GC declined rapidly in the early

neonatal period, and by 5–7 weeks of age only 0.004×10^6 OCT4⁺ GC per testis were present (with no OCT4⁺ GC identified in 4/9 animals), compared with 0.08×10^6 at 0.5 weeks of age (McKinnell et al., 2009). Thereafter, no OCT4⁺ GC were detected in any animals. VASA⁺ GC numbers increased progressively at each time-point, reaching 9.76×10^6 per testis at 22 weeks of age compared with 0.57×10^6 just after birth (McKinnell et al., 2009), a 17-fold increase. Our results confirm in quantitative terms the observations of Mitchell et al. (2008) that GC-expressing OCT4, and other undifferentiated GC markers such as AP2gamma and NANOG, become progressively less frequent during gestation and early post-natal life, such that their expression persists in only a few scattered GC at around 6 weeks of age. However, the present findings are in contrast to those of Albert et al. (2010) who reported that the numbers of OCT4⁺ and AP2gamma⁺ GC in marmosets remained unchanged at 8 weeks of age compared with newborn animals. Although a degree of individual variation between animals is well known in marmosets, we emphasize that OCT4⁺ GC are already undetectable in a proportion of animals at 5–7 weeks of age and that after this age we cannot detect OCT4⁺ GC in any animals. We note that the OCT4 antibody used by Albert et al. is reported to be capable of producing false-positive results in testis-derived cells, whereas the antibody used in the present study does not (Warthemann et al., 2012). It is possible that this factor, together with differences in counting methods, could account for the discrepancy between the two studies. Our present results also fit with the documented absence of OCT4⁺ GC in adult male marmosets (McKinnell et al., 2009; Albert et al., 2010), as in humans (Rajpert-de Meys et al., 2004).

Mitchell et al. (2008) reported that GC proliferation was present at all stages of fetal and early post-natal life examined in marmosets, although proliferation in the OCT4⁺ and the VASA⁺ subpopulations was not distinguished. In the present study, we found that OCT4⁺ and VASA⁺ GC proliferated at similar rates in the fetal marmoset testis. This was a surprising difference to the first and second trimester human fetal testis, in which GC proliferation occurs predominantly in the OCT4⁺ population (Mitchell et al., 2010). On the other hand, Honecker et al. (2004) reported the presence of proliferation in roughly equal numbers of undifferentiated and differentiated GC after 26 weeks of gestation in human pregnancy. In the first week after birth, overall GC proliferation in marmosets declined to less than half the rate during gestation, but again the PI for OCT4⁺ GC was comparable to VASA⁺ GC. This is also surprising, because OCT4⁺ GC decline rapidly in number between 0.5 and 2.5 weeks of age, while the VASA⁺ GC are expanding. It is possible either that OCT4⁺ GC lose expression of this marker during the proliferation cycle and instead become VASA⁺, or that continuing OCT4⁺ GC proliferation is counterbalanced by increased apoptosis in this subpopulation. Mitchell et al. (2008) showed that GC proliferation between 1 day and 6 weeks of post-natal life in marmosets varied between 9 and 14%. We have extended this to show that GC proliferation continues throughout early post-natal life. However, the PI reached a minimum of <3% at 14 weeks of age, around the reported end of mini-puberty (Lunn et al., 1994; McKinnell et al., 2001), before rising to ~7% at 22 weeks of age, when plasma testosterone levels are reported to be basal (Lunn et al., 1994; McKinnell et al., 2001). Together with our earlier study (Mitchell et al., 2008), these findings

indicate that the prepubertal primate testis is not truly quiescent (Chemes, 2001; Kelnar et al., 2002).

Although GC differentiation proceeds gradually during gestation in marmosets (Mitchell et al., 2008), the present study shows that the pace of GC differentiation is much more rapid in the perinatal period than previously supposed. The proportion of GC-expressing OCT4 declined from 54% in late fetal life to <0.5% at 2.5 weeks of age, and OCT4⁺ GC are absent by the time that mini-puberty is reported to end (Lunn et al., 1994; McKinnell et al., 2001). Our findings also suggest that the continuing expression of OCT4 in GC in neonatal marmosets represents a slower pace of differentiation in some individual cells, rather than evidence for the persistence of a stable subpopulation of pluripotent cells beyond early neonatal life, as suggested by Albert et al. (2010). In humans, a similar rapid and dramatic decline in the proportion of GC-expressing OCT4 was observed between fetal life and 4–5 weeks of age, suggesting that the progression of GC differentiation in perinatal humans and marmosets is similar. This interpretation is supported by the finding that GC OCT4 expression in boys disappears within a few months of birth (Rajpert-de Meys et al., 2004), i.e. by the end of mini-puberty in the human (Job et al., 1988; Gustafson et al., 1993). That OCT4⁺ GC disappear rapidly after birth is important, as maturational delay or failure to switch off expression of pluripotency factors in some GC in the human, may predispose such cells to becoming CIS/TGCC (Cools et al., 2005; Rajpert-de Meys, 2006; Hersmus et al., 2012). Our results suggest that the marmoset may be a useful animal model for perinatal GC development during the period when CIS cells are believed to arise.

A feature of early GC maturation in rodents is their migration within the seminiferous cords. Soon after birth the GC migrate from the centre of the cords to make contact with the basal membrane. This step occurs just prior to recommencement of proliferation, and is essential in order for further GC development to occur (Roosen-Runge and Leik, 1968; McGuinness and Orth, 1992; Nagano et al., 2000).

GC differentiation status in marmosets/humans has been described previously according to their position within the cord (Sharpe et al., 2003) or by their expression of pluripotency and/or differentiated GC markers (Gaskell et al., 2004; Mitchell et al., 2008; McKinnell et al., 2009; Albert et al., 2010). However, as far as we are aware there have been no quantitative studies in either species investigating the relationship between GC differentiation status and their migration/position. In the present study, we have described the position of both undifferentiated (OCT4⁺) and differentiated (VASA⁺) GC at several timepoints from late fetal until late neonatal life. We found that, during late fetal and early neonatal life, marmoset GC are heterogeneous in terms of their position, as well as in their differentiation marker expression (Mitchell et al., 2008; McKinnell et al., 2009). While OCT4⁺ GC were distributed roughly equally between basal and central locations in late gestation, the majority of VASA⁺ GC were centrally located, and this pattern remained unchanged immediately after birth. In the fetal human testis, GC were also heterogeneous with regard to position, but in contrast to the marmoset, VASA⁺ cells were predominantly basally located whereas the majority of OCT4⁺ cells were central. The significance of this species difference is unclear. We were unable to obtain data for GC position at other timepoints in the human due to the poor morphology of available tissue. We are therefore unable to establish whether VASA

expression precedes or follows the alignment of GC with the basal membrane in humans; however, Honecker *et al.* (2004) reported that the progressive relocation of GC to the basal membrane in the second trimester of pregnancy coincided with increasing intensity of VASA immunostaining.

The relative distribution of GC in marmosets changed markedly at 2.5 weeks after birth, when both OCT4⁺ and VASA⁺ cells were predominantly centrally located, and by 5–7 weeks 74% of OCT4⁺ cells and 80% of VASA⁺ cells were centrally located. It is striking that these changes occurred at the same time as OCT4 expression was rapidly declining. However, it is uncertain as to whether the change in position is directly related to GC differentiation status, or even whether it is due to active migration of the GC. In fetal life and just after birth, the Sertoli cells are dispersed throughout the seminiferous cords and intermingled with the GC, but later take up a more organized location, forming a ring just inside the perimeter of the cords. It may be that the relocation of GC towards the centre of the cords from 2.5 weeks of age is therefore simply due to their displacement by the Sertoli cells. The pattern remained unchanged until 22 weeks of age after the expected end of mini-puberty (Lunn *et al.*, 1994; McKinnell *et al.*, 2001). At this time the proportion of centrally located VASA⁺ GC declined to 40%, while the proportion basally located increased to 40%. In contrast to the marmoset and human, the seminiferous cords of the late fetal rat testis are much more organized and the GCs much more homogeneous in terms of position. At this age all GC are VASA⁺ and we found that almost 80% were centrally located and <3% basal. However, as expected, from 4 days after birth, GC migrated to make contact with the basal membrane and by 8 days of age more than 70% were basally located.

Spermatocytes do not appear until 32–35 weeks of age in the marmoset (de Souza *et al.*, 1988; Garde *et al.*, 1991; Kelnar *et al.*, 2002), indicating that GC migration to the basal lamina may be complete by that time, and more mature GC types are present by 43 weeks (Chandolia *et al.*, 2006). We therefore conclude that the movement of GC towards the cord periphery in marmosets at 22 weeks of age is analogous to the migration seen in the neonatal rat, and is a precursor of further GC differentiation. It is notable that this relocation, commencing between 14 and 22 weeks of age, occurs subsequent to the complete loss of GC OCT4 expression, just as it does in the rodent, albeit over a longer timespan. This may be significant, as both Honecker *et al.* (2004) and Cools *et al.* (2005) reported that OCT4⁺ GC in normal neonatal humans are almost exclusively located in the centre of seminiferous cords. In contrast, in boys with disorders of sexual development, who are at increased risk of TGCC, OCT4 expression persists in some GC and these are predominantly located adjacent to the basal membrane (Cools *et al.*, 2005; Hersmus *et al.*, 2012). GC identified as CIS, the precursor of TGCC, are always basally located (Gondos, 1993). We therefore speculate that loss of OCT4 expression prior to GC migration to the basal membrane is important, and that persistence of OCT4 expression in some GC, combined with their abnormal location in the basal niche may, in the human, predispose such GC to becoming CIS. The present findings in respect of marmoset GC location/migration strengthen our suggestion that the marmoset may represent a useful animal model for the study of the origins of CIS in humans.

In summary, our results show that GC differentiation and relocation in the perinatal marmoset testis is remarkably similar to that in the

human, and fundamentally different from that in the rat. Much of this difference may stem from the much more rapid entry of the rat into puberty and the resulting absence of any period of 'mini-puberty' as in the human and marmoset. In particular, the similarities in GC differentiation in the early post-natal period in the marmoset and the human indicate that the marmoset may be a particularly useful model for studying the regulation of perinatal GC differentiation, which in the human has direct relevance to the development of CIS, and thereby TGCC.

Authors' roles

C.M.: Data collection, analysis and interpretation, manuscript preparation. R.T.M., R.M.S.: Conception and design, critical contributions to manuscript. K.M.: Fetal and neonatal marmoset testis tissue. R.A.A.: Fetal human testis tissue. C.J.H.K., W.H.W.: Critical contributions to manuscript.

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Conflict of interest

None declared.

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